

REMARKS**Status of the Claims:**

Claims 1 and 3-65 are pending in this application and claims 6, 7, 10, 21-36, 41-58 and 62-65 are withdrawn. Claims 3 and 38 are cancelled herein without waiver or prejudice to Applicants' right to pursue claims to inventions claimed in the cancelled claims in continuant applications claiming priority to the present application.

Claims 1, 4-7, 11-12, 14-20, 25-27, 37, 39-40 and 59-60 are amended herein. No new matter is introduced.

Claims 1, 16, 37, 59 and 60 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing new matter. Claims 1, 3, 4-9, 11-28, 37-40 and 59-61 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. Claims 1, 3-5, 8, 9, 11-28, 37-40 and 59-61 have also been rejected under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite.

Explanation of Amendments:

Applicants have amended the specification in response to the Examiner's concerns regarding the use of trademarks and abbreviated terms. Support for these amendments to the specification is found throughout the application as originally filed or could be readily ascertained by one of ordinary skill in the art from Applicants' original disclosure. Specifically, the term "DCC" identified by the Examiner has been amended to recite "dicyclohexylcarbodiimide (DCC)." As shown by an entry from the NIST Standard Reference Database (Exhibit 1), the term DCC is known in the art as dicyclohexylcarbodiimide.

In this paper, Applicants have amended claim 1 to clarify the invention. Claim 1 now recites the following:

1. A polysaccharide-protein conjugate or an oligosaccharide-protein conjugate comprising:
a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide covalently attached to protein via a β -propionamido linkage,
wherein said de-N-acetylated polysaccharide or said de-N-acetylated oligosaccharide is derived from bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide, naturally or synthetically obtained,
wherein the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the polysaccharide or the oligosaccharide,
wherein the degree of de-N-acetylation is at least 50%, and
wherein the protein is bacterial protein or synthetic protein comprising a lysine residue or a cysteine residue.

Support for this revised version of claim 1 is found throughout the application as originally filed. Support for “a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide covalently attached to a protein via a β -propionamido linkage” is found, for example, on page 10, lines 6-14 of the specification (“... first treated hydrolyzed using base or enzyme to remove part or all of its N-acyl groups... then directly coupled to protein ... to form an immunogenic β -propionamido-linked polysaccharide-protein conjugate”) and Figure 1 (showing hydrolysis of compound (1) to obtain compound (2)) as originally filed. Support for “said de-N-acetylated polysaccharide or said de-N-acetylated oligosaccharide is derived from bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide, naturally or synthetically obtained” is found, for example, on page 5, line 20 to page 6 line 32 of the specification. (“Polysaccharides or oligosaccharides may be obtained from a variety of sources including gram-negative, gram-

positive bacteria, yeast, cancer cells or recombinant forms of each....”). Support for “the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the polysaccharide or the oligosaccharide” is found, for example, on page 12, lines 2-6 of the specification (“... the isolated β -propionamido-linked polysaccharide-protein conjugates described above may be used as an antigen to generate antibodies that are reactive against the polysaccharide or oligosaccharide and hence reactive against the organism or cell from which the polysaccharide or oligosaccharide was isolated.”). Support for “the degree of de-N-acetylation is at least 50%” is found, for example, on page 6, lines 12-13 of the specification (“... at least about 50% of the N-acetyl groups are removed by hydrolysis....”). Support for “wherein the protein is bacterial protein or synthetic protein comprising a lysine residue or a cysteine residue” is found, for example, on page 10, lines 3-5 of specification (“The point of attachment are between lysine or cysteine residues of the protein and the N-acryloyl groups of the polysaccharide or oligosaccharide.”).

Claims 4, 5, 6, 7, 11, 12, 15, 37 and 40 have been amended to add the word “the” to further clarify the invention.

Claims 4 and 5 have been amended to add the element “a capsular polysaccharide.” Support for these amendments can be found, for example, from page 6, lines 18-32 of the specification.

Claim 14 has been amended to recite “wherein the β -propionamido linkage comprises a β -carbon attached to: (1) a side-chain nitrogen of the lysine residue of the protein, or (2) a sulfur of the cysteine residue of the protein.” Support for this amendment is found, for example, on page 10, lines 3-5 of specification and in Figure 1 of the drawings as originally filed.

In this paper, Applicants have amended independent claim 16 to clarify the invention. Independent claim 16 now recites the following:

16. A polysaccharide-protein conjugate or an oligosaccharide-protein conjugate produced by a method comprising:

A) de-N-acetylating a bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide, naturally or synthetically obtained, by at least 50% using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide with an acryloylating reagent to form an N-acryloylated polysaccharide or an N-acryloylated oligosaccharide, and

C) reacting the N-acryloylated polysaccharide or the N-acryloylated oligosaccharide with protein to form a β -propionamido linkage,

wherein the protein is bacterial protein or synthetic protein comprising a lysine residue or a cysteine residue, and

wherein the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the polysaccharide or the oligosaccharide.

Support for this revised version of claim 16 is found throughout the application as originally filed. Support for “de-N-acetylating a bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide, naturally or synthetically obtained, by at least 50% using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide” is found, for example, on page 10, lines 6-14 of the specification (“... first treated hydrolyzed using base or enzyme to remove part or all of its N-acyl groups....”), on page 6, lines 12-13 of the specification (“... at least about 50% of the N-acetyl groups are removed by hydrolysis....”), on page 5, line 20 to page 6 line 32 of the specification, and in Figure 1

(showing hydrolysis of compound (1) to obtain compound (2)) as originally filed. Support for “N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide with an acryloylating reagent to form an N-acryloylated polysaccharide or an N-acryloylated oligosaccharide” is found, for example, on page 8, lines 30-31 of the specification. Support for “reacting the N-acryloylated polysaccharide or the N-acryloylated oligosaccharide with protein to form a β -propionamido linkage” is found, for example, on page 10, lines 6-14 of the specification (“... then directly coupled to protein ... to form an immunogenic β -propionamido-linked polysaccharide-protein conjugate”) and Figure 1 as originally filed. Support for “wherein the protein is bacterial protein or synthetic protein comprising a lysine residue or a cysteine residue” is found, for example, page 10, lines 3-5 of specification (“The point of attachment are between lysine or cysteine residues of the protein and the N-acryloyl groups of the polysaccharide or oligosaccharide.”). Support for “the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the polysaccharide or the oligosaccharide” is found, for example, on page 12, lines 2-6 of the specification (“... the isolated β -propionamido-linked polysaccharide-protein conjugates described above may be used as an antigen to generate antibodies that are reactive against the polysaccharide or oligosaccharide and hence reactive against the organism or cell from which the polysaccharide or oligosaccharide was isolated.”).

Support for the amendments to claims 18-20 is found, for example, on page 10, lines 6-25 of the specification.

Support for the amendments to claims 26, 27 and 37 is found, for example, on page 12, lines 2-6 of the specification.

Claim 39 was amended to depend from claim 37 instead of claim 38, which is now cancelled. Prior to the filing of this paper, claim 39 depended from claim 38, which depended from claim 37.

Support for the amendments to claims 25 and 40 is found, for example, on page 1, line 25 of the specification.

Support for claims 59 and 60 can be found, for example, from page 8 line 24 to page 9, line 7 of the original specification as filed.

Objections to the Specification:

The specification is objected to by the Examiner. Applicants' respectfully request reconsideration and withdrawal of the objections, in view of the following:

(a) The Examiner objects to the specification because the first paragraph of the specification as amended allegedly does not accurately reflect current status of the prior non-provisional application. (Office Action, 4). Under 35 U.S.C. §120, Applicants are only obligated to amend the specification to contain a "specific reference to the earlier filed application." According to MPEP 201.11, III, A, "[a]n example of a proper benefit claim is 'this application is a continuation of prior Application No. ---, filed ---.'" In accordance with 35 U.S.C. §120, the first paragraph of the specification as amended by the Preliminary Amendment of January 20, 2004 provides a specific reference to the earlier filed applications and indicates the relationship between the present Application and the earlier filed applications. In fact, the first paragraph of the specification as amended by the Preliminary Amendment of January 20, 2004 closely follows the example provided in the MPEP. Nonetheless, to expedite the prosecution of this application and for the Examiner's convenience, Applicants have amended

the first paragraph of the specification to include the current “now abandoned” status of prior application Serial No. 09/376,911.

(b) The Examiner objects to the specification allegedly because the recitation of trademarks should be capitalized wherever it appears. The Office Action suggests “that Applicants examine the whole specification to make similar corrections to trademark recitations, wherever such recitations appear.” (Office Action, 5). To expedite the prosecution of this application, Applicants have amended the specification to reflect trademark recitations, as suggested by the Examiner.

(c) The Examiner objects to the specification because the claim elements “N-propionated polysaccharide” and “N-propionated oligosaccharide” allegedly lack “clear support or antecedent basis in the specification.” (Office Action, 5). In view of the amendments presented herein, Applicants respectfully submit that this objection is moot.

(d) The Examiner objects to the specification because certain abbreviated terminologies are allegedly not understood. Specifically, the Examiner objects to the term “DCC” on page 9, line 1. For the Examiner’s convenience, Applicants have amended the specification to more particularly point out certain abbreviated terms as such terms would be readily understood by one of ordinary skill in the art from the Applicants’ original disclosure.

For at least the foregoing amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the objections to the specification.

Rejection(s) under 35 U.S.C. §112, First Paragraph (New Matter):

Claims 1, 16, 59 and 60 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing new matter because “there is no support in these parts of the specification

for a polysaccharide- or oligosaccharide-protein conjugate as claimed, comprising an N-propionated polysaccharide or N-propionated oligosaccharide... directly coupled to a protein through 'β-position sites of one or more propionate moieties'... having at least 50% the N-propionated polysaccharide or N-propionated oligosaccharide de-N-acetylated or having at least 95% of the N-acryloylated polysaccharide or oligosaccharide, wherein the conjugate elicits 'protective' antibodies reactive with the N-propionated polysaccharide or the N-propionated oligosaccharide." (Office Action, 6). In view of the amendments to claims 1, 16, 59 and 60, however, Applicants respectfully assert that this ground of rejections is moot.

Claim 37 stands rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing new matter because "there appears to be no descriptive support in the specification as originally filed" for the claim element "protective immunity against at least one member of a genus of an organism from which the polysaccharide or oligosaccharide component of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate was obtained." (Office Action, 7). *Ipsis verbis* support is not necessary to support a claim and the specification clearly provides ample support throughout the application as originally filed for the above-identified claim element. See MPEP 2163, II, 3(a) (*citing Vas-Cath*, 935 F.2d 1555, 1563 (Fed. Cir. 1991) and *Martin v. Johnson*, 454 F.2d 746, 751 (CCPA 1972)). Specifically, support can be found, for example, on page 4, lines 29-31, page 11, lines 22-25 and page 12, lines 1-17 of the specification and claim 37 as originally filed.

Rejection(s) Under 35 U.S.C. §112, First Paragraph (Enablement):

Claims 1, 3, 4-9, 11-28, 37-40 and 59-61 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement.

The Office Action rejects claims 18 and 20 under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement because the specification allegedly “does not reasonably provide enablement for such a conjugate wherein the coupling is conducted **at a pH of 7.0 and in a phosphate buffer**, wherein the the conjugate ‘elicites protective antibodies’ reactive with the N-propionated polysaccharide or N-propionated oligosaccharide.... (Office Action, 7)(*emphasis added*.). It appears that the Office Action inadvertently combined the “at a pH of about 7.0” element from claim 18 with the “phosphate buffer” element of claim 20. Applicants respectfully submit that this combined construction of claims 18 and 20 is improper. Nonetheless, Applicants address the Examiner’s enablement rejections of claims 18 and 20 below.

It is well-established that “[c]ompliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed ... The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation.” MPEP2164.02. Nonetheless, the Office Action improperly imposes an “actual reduction to practice requirement.” *See* MPEP 2164.02 (“An applicant need not have actually reduced the invention to practice prior to filing.”). Specifically, the Examiner contends that “there is no showing that a bacterial or non-bacterial N-acryloylated polysaccharide or a bacterial or non-bacterial N-acryloylated oligosaccharide, including an N-acryloylated GBS polysaccharide or oligosaccharide, **is successfully conjugated** to a protein by Michael addition at a pH of about ‘about 7.0’ or in a phosphate buffer reagent such that the resulting conjugate ‘elicits protective antibodies with the N-propionated polysaccharide or N-propionated oligosaccharide.’” (Office Action, 8-9)(*emphasis added*.).

Applicants claims 18 and 20 are not limited to conjugates formed by Michael addition. Rather, the claims are directed to any conjugate wherein an N-acryloylated polysaccharide or an N-acryloylated oligosaccharide is reacted (by Michael addition or by any other mechanism) with protein to form a β -propionamido linkage. In fact, the specification as originally filed expressly provides that “[i]n another embodiment, the method of conjugation is conducted at a neutral pH of about 7.0 for optimal reactivity of thiol (SH) groups of cysteine residues of the protein.” (Specification, page 10, lines 17-19). In addition, the specification teaches that

[t]he selection of pH for conducting the method of conjugation may be based on the number of reactive groups in a particular carrier protein. For example, a method using a protein composed of more reactive lysine residues as compared to cysteine residues is preferably conducted at a basic pH. A method of conjugation using a protein composed of more reactive cysteine residues as compared to lysine residues is preferably conducted at about a neutral pH. (Specification, page 10, lines 19-25).

The specification also provides examples of buffered reagents which include “carbonate/bicarbonate, borate buffer, phosphate and the like.” (Specification, page 10, lines 27-28). Accordingly, the Applicants’ specification provides ample guidance to one of ordinary skill in the art to determine the proper pH and buffered reagents for conducting the method of conjugation without undue amount of experimentation.

“The enablement requirement refers to the requirement of 35 U.S.C. 112, first paragraph that the specification describe how to make and how to use the invention.” MPEP 2164. “But because only an enabling disclosure is required, applicant need not describe all actual embodiments.” MPEP 2164.02. The Examiner contends that “[a]ssuming that the Michael reaction does take place to some extent at pH 7.0, it is unlikely that the resultant poorly

conjugated polysaccharide or poorly conjugated oligosaccharide would elicit ‘protective antibodies.’” (Office Action, 9-10). As discussed above, Applicants’ claims encompass any mechanism for forming the conjugates and is not limited to Michael addition. Furthermore, the specification provides a particular embodiment for thiol (SH) groups with an **optimal reactivity** at a neutral pH of about 7.0. (See Specification, page 10, lines 17-19). Contrary to that suggested by the Office Action, an “optimal reactivity” would hardly result in a “poorly conjugated polysaccharide” or “poorly conjugated oligosaccharide.”

The Office Action rejects claims 1, 3, 4-9, 11-28, 37-40 and 59-61 under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner contends that “[a] myriad of pathogenic organism including pathogenic bacteria, and any number of serogroups, serotypes, or species of *Streptococcus*, and any types of Group B *Streptococci* are encompassed within the scope of the claims against which the claimed polysaccharide- or oligosaccharide-protein conjugate vaccine is required to elicit ‘protective antibodies’ to.” (Office Action, 11). Contrary to the Examiner’s position, claims 1 and 16, as amended, are directed to conjugates that elicit protective antibodies reactive with a bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide, naturally or synthetically obtained. As claimed, an exemplary conjugate of the present invention having a Group B *Streptococci* polysaccharide component would elicit antibodies reactive with that particular Group B *Streptococci* polysaccharide.

The Examiner also takes the position that “[n]either there is evidence, nor is it predictable that a non-capsular polysaccharide, for example, of GBS III when conjugated as described in the instant application, would elicit ‘protective antibodies’ to *Streptococci* of heterologous capsular types, absent a concrete showing.” (Office Action, 12). Contrary to the

Examiner's position, Applicants' specification teaches one of ordinary skill in the art how to make and use the present invention as claimed. Notably, the specification provides that "[i]n certain cases the polysaccharide used with this invention may induce antibody which is cross-reactive with other pathogenic organisms and thus have ability in protecting against infection by these other bacteria." (Specification, page 15, lines 29-31). In view of Applicants' specification, one of ordinary skill in the art would understand that to elicit a cross-reactive antibody using a conjugate of the present invention, the polysaccharide component of the conjugate should itself be select from polysaccharides capable of inducing an antibody that is cross-reactive with other pathogenic organisms. Such cross-reactive polysaccharides can be easily determined by those of ordinary skill in the art without any undue experimentation. *See e.g.*, Ota et al., "Immunological Study of Cross-Reactive Polysaccharide Antigens (Types a, d, and h) of Oral Streptococcus spp. with Monoclonal Antibodies," Infection and Immunity, 55(1), 266-268 (1987)(Exhibit 2).

The Office Action rejects claims 25 and 40 under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner contends that "it is neither shown within the instant specification, nor is it predictable that the instantly claimed GBS conjugate when combined with a preparation containing DTaP or Hib conjugate ... would not result in an immune response-suppressing effect on one or more vaccine components." (Office Action, 14).

"All questions of enablement are evaluated against the **claimed** subject matter." MPEP 2164.08 (emphasis added.). Applicants' claims 25 and 40 are directed to a combination of a polysaccharide-protein conjugate or a oligosaccharide-protein conjugate with a second immunogenic component selected from the group of immunogens consisting of diphtheria-tetanus-pertussis (DTP), diphtheria-tetanus-acellular pertussis (DTaP), tetanus-diphtheria (Td),

diphtheria-tetanus-acellular pertussis-*Haemophilus influenzae type b* (DTaP-Hib), diphtheria-tetanus-acellular pertussis-inactivated poliovirus-*Haemophilus influenzae type b* (DTaP-IPV-Hib), and combinations thereof. Applicants' claims 25 and 40 do not require the combination of a conjugate with a secondary immunogenic component to provide the same or greater immune response as the two immunogenic components administered separately. Rather, Applicants' claims require that the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the bacterial, yeast or cancer cell surface or capsular polysaccharide or oligosaccharide. The Examiner has not shown that the addition of a second immunogenic component would cause the conjugates of the present invention to be unreactive with the polysaccharide or the oligosaccharide. On the contrary, the Eskola reference cited by the Examiner shows that Hib antibodies were obtained (*i.e.*, 0.37 µg/mL and 0.56 µg/mL) when DTP-a was administered in combination with Hib capsular polysaccharide, showing that the polysaccharide remains reactive even in the presence of DTP-a.

For at least the foregoing amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the enablement rejections to claims 1, 3, 4-9, 11-28, 37-40 and 59-61.

Rejections Under 35 U.S.C. §112, Second Paragraph:

Claims 1, 3-5, 8, 9, 11-28, 37-40 and 59-61 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite, for allegedly failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully request reconsideration and withdrawal of the rejection of these claims, in view of the following:

(a) The Office Action rejects claims 1, 14 and 16 for allegedly lacking proper antecedent basis for the element “N-propionated oligosaccharide.” Claims 1, 14 and 16 have been amended and no longer recite “N-propionated oligosaccharide.”

(b) The Office Action rejects claims 1, 3-5, 11, 12, 15, 17, and 37 for allegedly lacking proper antecedent basis for the element “oligosaccharide.” Claims 1, 4-5, 11, 12, 15, 17, and 37 have been amended to recite “the oligosaccharide” and claim 3 has been canceled.

(c) The Office Action rejects claims 37 and 40 for allegedly lacking proper antecedent basis for the element “oligosaccharide-protein conjugate.” Claim 37 has been amended to no longer recite “oligosaccharide-protein conjugate” and claim 40 has been amended to recite “the oligosaccharide-protein conjugate.”

(d) The Office Action rejects claims 25 and 40 for allegedly being incorrect in identifying “Hib” as “Haemophilus influenzae type B.” Claims 25 and 40 have been amended to recite “Haemophilus influenzae type b.”

(e) The Office Action rejects claims 1 and 16 for allegedly being unclear that the N-propionated polysaccharide- or N-proionated oligosaccharide-reactive antibodies are protective against. Claims 1 and 16 have been amended to indicate that the polysaccharide-protein conjugate or the oligosaccharide-protein conjugate elicits protective antibodies reactive with the polysaccharide or the oligosaccharide.

(f) The Office Action rejects claims 26 and 27 as allegedly being indefinite and/or improperly broadening the scope in the element “an N-propionated polysaccharide-specific” or “an N-propionated oligosaccharide-specific” immun response or immunoglobulin. Claims 26 and 27 have been amended and no longer recite “N-propionated polysaccharide-specific” or “N-propionated oligosaccharide-specific.”

(g) The Office Action rejects claim 16 as allegedly being vague, indefinite and internally inconsistent in the scope of the limitation protein. Claim 16 has been amended to recite “wherein the protein is bacterial protein or synthetic protein....”

(h) The Office Action rejects claim 26 because it allegedly has improper antecedent basis in the limitations “the conjugates according to any one of claims 1 and claim 16.” Claim 26 has been amended to recite “[a]n immunogen comprising the conjugate according to any one of claim 1 and claim 16....”

(i) The Office Action rejects claims 3-5, 8, 9, 11-15, 17-28, 37-40 and 59-61 because they depend directly or indirectly from claim 1 or 16. Applicants have addressed the Examiner’s concerns to claims 1 and 16 in preceding sections.

On the basis of the foregoing amendments and remarks, Applicants respectfully request withdrawal of the rejection of claims 1, 3-5, 8, 9, 11-28, 37-40 and 59-61 under 35 U.S.C. § 112, second paragraph.

Objections to Claims 1 and 16:

The Examiner objects to claims 1 and 16 for the use of “;” in lines 7 and 9 of claim 1 and line 4 of claim 16. The Examiner suggests “that Applicants replace it with a comma.” To expedite the prosecution of this application and for the Examiner’s convenience, claims 1 and 16 have been amended to remove the semicolons (“;”) therein and use commas (“,”) as suggested by the Examiner.

For at least the foregoing amendments and remarks, Applicants respectfully request reconsideration and withdrawal of the objections to claims 1 and 16.

CONCLUSION

Based on the foregoing remarks, Applicants respectfully request withdrawal of the rejections of claims and allowance of this application. In the event that a telephone conference would assist in the examination of this application, Applicants invite the Examiner to contact the undersigned at the number provided.

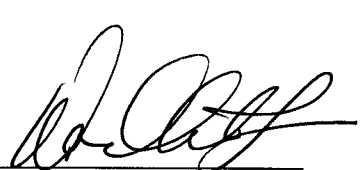
AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. 50-3732, Order No. 13564-105037. In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. 50-3732, Order No. 13564-105037.

Respectfully submitted,
King & Spalding, LLP

Dated: November 30, 2007

By: _____


Kenneth H. Sonnenfeld / Wan Chieh Lee
Reg. No. 33,285 / Reg. No. 57,297

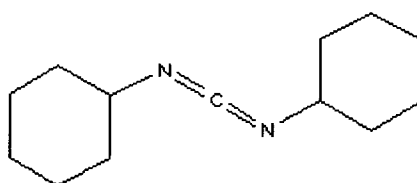
Customer Number 65989
Correspondence Address:

King & Spalding
1185 Avenue of the Americas
New York, NY 10036-4003
(212) 556-2324 Telephone
(212) 556-2222 Facsimile

EXHIBIT 1

Methanediimine, n,n'-dicyclohexyl-

- **Formula:** C₁₃H₂₂N₂
- **Molecular weight:** 206.33
- **IUPAC International Chemical Identifier:**
 - InChI=1/C13H22N2/c1-3-7-12(8-4-1)14-11-15-13-9-5-2-6-10-13/h12-13H,1-10H2
 - Download the identifier in a file.
- **CAS Registry Number:** 538-75-0
- **Chemical structure:**



This structure is also available as a 2d Mol file .

- **Other names:** Dicyclohexylcarbodiimide; Cyclohexanamine, N,N'-methanetetraylbis-; 1,3-Dicyclohexylcarbodiimide; Carbodiimide, dicyclohexyl-; Carbodicyclohexylimide; DCC; DCCD; DCCI; N,N'-Dicyclohexylcarbodiimide; N,N'-Methanetetraylbiscyclohexanamine
- **Information on this page:**
 - Notes / Error Report
- **Other data available:**
 - IR Spectrum
 - Mass Spectrum
- **Options:**
 - Switch to calorie-based units

Notes / Error Report

Go To: Top

- Data from NIST Standard Reference Database 69, June 2005 Release: *NIST Chemistry WebBook*
- The National Institute of Standards and Technology (NIST) uses its best efforts to deliver a high quality copy of the Database and to verify that the data contained therein have been selected on the basis of sound scientific judgment. However, NIST makes no warranties to that effect, and NIST shall not be liable for any damage that may result from errors or omissions in the Database.
- If you believe that this page may contain an error, please fill out the error report form for this page.

© 1991, 1994, 1996, 1997, 1998, 1999, 2000, 2001, 2003, 2005 copyright by the U.S. Secretary of Commerce on behalf of the United States of America. All rights reserved.

NIST

National Institute of Standards and Technology

Standard Reference
Data ProgramData
GatewayOnline
DatabasesChemist
WebBook

If you have comments or questions about this site, please contact us.

EXHIBIT 2

Immunological Study of Cross-Reactive Polysaccharide Antigens (Types a, d, and h) of Oral *Streptococcus* spp. with Monoclonal Antibodies

FUSAO OTA,* HIROHISA KATO, AND KOMEI FUKUI

Department of Oral Microbiology and Immunology, School of Dentistry, The University of Tokushima,
Tokushima 770, Japan

Received 13 August 1986/Accepted 17 October 1986

Two monoclonal antibodies against the cross-reactive antigens of *S. cricetus* (type a) and *S. sobrinus* (type d) were isolated. Galactose and especially melibiose inhibited the precipitin reaction markedly. Inhibition by melibiose was over 200-fold stronger than that by galactose. This may indicate that galactose- α 1,6-glucose is the predominant antigenic determinant of this cross-reactive antigen. This antigen was also found in *S. sobrinus* type h strains, but no antigen was found in one type d strain (OMZ176).

The ability of *Streptococcus mutans* to form dental caries is well documented (5, 7, 8, 16). *S. mutans* was the original designation for the members of the mutans group of streptococci, but the subspecies of *S. mutans* have been elevated to species level and approved (4, 11, 17). They have traditionally been divided into eight serological groups, whereas serotypes c, e, and f remain known as *S. mutans*, type a is now designated *S. cricetus*, types d, g, and h are *S. sobrinus*, and type b is *S. rattus*. As the cell surface structure may be an important factor in the cariogenicity of these mutans group streptococci, many studies of cell surface polysaccharide antigen have been described (3, 8-10, 12, 13, 15, 18). It has been reported by Mukasa and Slade (12) that the *S. cricetus* (type a) and *S. sobrinus* (type d) cross-reactive antigenic (a-d) site is present in cell wall polysaccharide. They showed by precipitin inhibition tests that a terminal galactose is responsible for the a-d site. On the other hand, Brown and Bleiweis (3) concluded that galactose linked α (1→6) to a subterminal sugar moiety is specific for the a-d site.

In this study, we analyzed this cross-reactive antigen with two monoclonal antibodies (MAbs) specific for the a-d site and compared the characteristics of these two MAbs.

Bacterial strains were maintained in brain heart infusion agar (Difco Laboratories, Detroit, Mich.). The cells were grown in brain heart infusion medium supplemented with 0.4% glucose for 18 h at 37°C. MAbs were prepared principally by the procedure of Galfre et al. (6, 9). BALB/c mice were immunized intraperitoneally with 0.5 ml of live *S. cricetus* HS1 (type a) cells (optical density, 1.0 at 660 nm) in Freund complete adjuvant. After 1 month, mice were immunized with the same type of cell in Freund incomplete adjuvant. After another month, a final intravenous injection of bacterial cells in phosphate-buffered saline (pH 7.5; 0.2 ml; optical density, 1.0 at 660 nm) was carried out. Three days later, spleen cells were prepared for hybridization. Spleen cells were hybridized with Sp2/0-Ag14 myeloma cells in the presence of 50% polyethylene glycol 1500 (M. A. Bioproducts, Walkersville, Md.). Antibody-producing cells were cloned at least twice by the limiting dilution method. Antibody-producing cells were detected by radioimmunoassay as described before (14). Bacterial cells were reacted

with a culture fluid for 30 min at room temperature. The cells were washed in phosphate-buffered saline by centrifugation and then reacted with 125 I-labeled goat anti-mouse immunoglobulin G or M (Cooper Biomedical, Inc., West Chester, Pa.). Antigen was obtained by autoclaving HS1 (type a) cells in saline (120°C, 20 min). DEAE-Sephadex A25, Sephadex G-200, and carboxymethyl Sephadex C25 columns were used for antigen purification as described by Mukasa and Slade (12). The chemical composition of antigen was nearly the same as that reported by Mukasa and Slade (12). Total sugar content (by weight) was 77.2% by the phenol-sulfuric acid method (9). Polysaccharide was composed of 16.6% glucose and 73.9% galactose by weight; this was determined with Glucostat (Worthington Diagnostics, Freehold, N.J.) and galactose UV test (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) reagents, respectively, as described by van de Rijn et al. (19). No detectable amount of protein was found by the method of Lowry et al. (9). MAbs, which were found to be of immunoglobulin class M by radioimmunoassay, were obtained from ascitic fluids of pristane-primed BALB/c mice. A 30 to 50% ammonium sulfate-precipitable fraction was used for analysis. The immunodiffusion tests and precipitin reaction tests used have been previously described (9).

Two MAbs, designated a-4 and a-84, were isolated, and their reactivity was determined by radioimmunoassay (Table 1). The MAbs reacted with the cells of HS1 and E49 (*S. cricetus*, type a), MT615R and B13 (*S. sobrinus*, type d), and MF25 and BFe12 (*S. sobrinus*, type h). The MAbs didn't react with the other strains listed in Table 1. Unexpectedly, OMZ176 (*S. sobrinus*, type d) cells didn't react with the MAbs. Heterogeneity may exist in type d strains. Type h strains reacted with the MAbs. Type h strains were recently isolated and found to be closely related to *S. sobrinus* strains (2, 11). This cross-reactivity seems to be reasonable. These reactivities of MAbs were also confirmed with double-diffusion tests with autoclaved extracts (data not shown). Takada et al. (18) reported the existence of a-d-g and some other cross-reactive sites. The a-d-h site reported here seems to be different from the one reported by them.

Figure 1 shows the quantitative precipitin curves between the two MAbs and HS1 antigen. The equivalence point was reached with 40 μ g of purified HS1 antigen per 40 μ l of MAb a-4 and with approximately 100 μ g of the same antigen per 50

* Corresponding author.

TABLE 1. Reactivity of MABs with various strains of mutans group streptococci

Strain	Serotype	Mean (\pm SD) % reactivity ^a	
		MAB a-4	MAB a-84
<i>S. cricetus</i>			
HS1	a	100.0 \pm 7.7	100.0 \pm 12.7
E49	a	123.8 \pm 4.9	119.0 \pm 10.1
<i>S. rattus</i>			
FA1	b	9.0 \pm 2.4	0.0 \pm 8.4
BHT	b	5.4 \pm 1.3	0.8 \pm 2.0
<i>S. mutans</i>			
Ingbritt	c	2.3 \pm 3.1	0.5 \pm 4.6
MT6R	c	3.4 \pm 0.6	0.0 \pm 0.3
LM7	e	1.0 \pm 4.0	0.0 \pm 2.3
P4	e	5.9 \pm 2.2	0.0 \pm 2.0
MT703	e	4.7 \pm 0.7	0.8 \pm 5.8
SE17	f	2.3 \pm 1.4	2.6 \pm 3.3
OMZ175	f	5.1 \pm 1.1	0.8 \pm 2.4
MT557	f	7.9 \pm 1.6	0.0 \pm 2.0
<i>S. sobrinus</i>			
OMZ176	d	8.1 \pm 0.8	7.2 \pm 0.6
MT615R	d	105.0 \pm 38.5	80.0 \pm 6.8
B13	d	94.6 \pm 0.8	96.0 \pm 4.0
OMZ65	g	5.2 \pm 3.4	5.5 \pm 5.2
6715	g	6.3 \pm 3.8	0.5 \pm 4.5
K1R	g	7.0 \pm 0.2	1.8 \pm 6.8
MF25	h	131.5 \pm 11.7	80.7 \pm 12.0
BFe12	h	119.5 \pm 9.7	105.4 \pm 12.3

^a The reactivity of strain HS1 cells with each MAB was taken as 100%. Results of two experiments. SD, Standard deviation.

μ l of MAB a-84. These conditions were used in precipitin inhibition tests. It was reported by Mukasa and Slade (12) that galactose, glucose, galactosamine, and glucosamine are contained in hot-water extracts of type a strains, and a terminal galactose is responsible for the a-d site according to precipitin inhibition tests. Brown and Bleiweis (3) determined that galactose and glucose are present in type a

TABLE 2. Inhibition of the precipitin reaction between strain HS1 (type a) antigen and MABs

Inhibitor ^a	Mean (\pm SD) ^a % inhibition	
	MAB a-4	MAB a-84
Glucose	1.8 \pm 4.3	7.6 \pm 0.8
Rhamnose	4.8 \pm 6.2	13.1 \pm 0.6
Galactose	43.6 \pm 0.9	44.7 \pm 3.8
α -Methylgalactopyranoside	8.1 \pm 6.6	43.0 \pm 2.3
β -Methylgalactopyranoside	4.6 \pm 5.7	3.0 \pm 6.4
N-Acetylgalactosamine	2.9 \pm 6.6	0.0 \pm 4.5
N-Acetylglucosamine	0.9 \pm 3.7	3.6 \pm 3.7
Melibiose (Gal ^{1,6} Glc)	100.0 \pm 0.1	100.0 \pm 1.0
Raffinose (Gal ^{1,6} Glc ^{1,2} Fru)	98.2 \pm 0.9	97.0 \pm 2.6
Stachyose (Gal ^{1,6} Glc ^{1,6} Glc ^{1,2} Fru)	2.0 \pm 1.7	98.0 \pm 8.5
Lactose (Gal ^{1,4} Glc)	3.7 \pm 0.8	12.1 \pm 1.7
Sucrose (Fru ^{1,2} Glc)	2.0 \pm 1.8	5.2 \pm 9.6
Isomaltose (Glc ^{1,6} Glc)	0.0 \pm 7.1	1.7 \pm 2.2
Cellobiose (Glc ^{1,4} Glc)	5.8 \pm 1.9	9.5 \pm 1.7
Maltose (Glc ^{1,4} Glc)	4.6 \pm 0.3	4.8 \pm 5.8
Gentiobiose (Glc ^{1,6} Glc)	0.0 \pm 6.4	19.9 \pm 4.4

^a The abbreviations used for galactose, glucose, and fructose are Gal, Glc, and Fru, respectively. A total of 2.5 μ mol of inhibitor was used for MAB a-4, and 1.25 μ mol was used for MAB a-84. The results are from two experiments.

^b SD, Standard deviation.

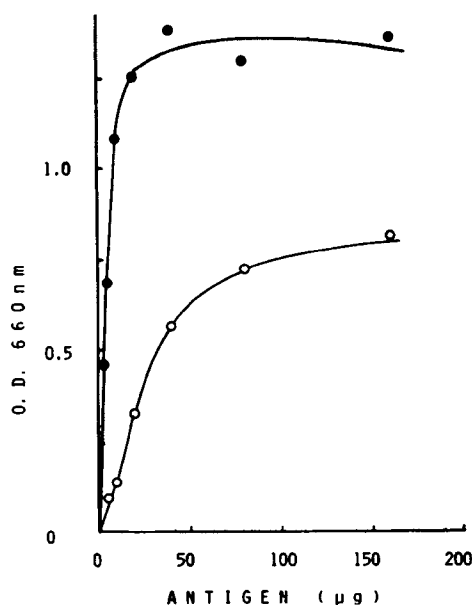


FIG. 1. Quantitative precipitin curves between two MABs and purified HS1 antigen. Samples of 40 μ l of MAB a-4 (●) and 50 μ l of MAB a-84 (○) were reacted with increasing amounts of antigen. The final volume was adjusted to 120 μ l. After incubation at 37°C for 1 h, the mixture was held at 4°C for 1 day. The amount of protein precipitated was determined colorimetrically. O.D., Optical density.

polysaccharide antigen by hot-formamide extraction. They also demonstrated by precipitin inhibition tests that galactose was the strongest monosaccharide inhibitor (38%) among monosaccharides and that melibiose and gentiobiose were the strongest disaccharide inhibitors (47%). It was also reported that galactose and glucose are mainly contained in type d and h cell wall polysaccharides (10, 13, 15).

To determine which sugars are responsible for the precipitin reaction, quantitative precipitin inhibition tests were performed with the two MABs (Table 2). The inhibition patterns of the two MABs were almost the same. This indicated that both MABs recognize the same site of the antigen. Among monosaccharides, galactose showed the strongest inhibition, as reported before (3, 12). α -Methylgalactoside inhibited the reaction of MAB a-84 but not that of MAB a-4. Melibiose inhibited the reaction of both MABs

TABLE 3. Inhibition of the precipitin reaction between HS1 antigen and MABs at high doses

Inhibitor	Amt (μ mol)	Mean (\pm SD) ^a % inhibition	
		MAB a-4	MAB a-84
Rhamnose	20	0	0
Glucose	20	0	56.1 \pm 4.9
	10		20.1 \pm 3.9
α -Methylglucoside	20	0	80.8 \pm 2.9
β -Methylglucoside	20	16.5 \pm 0.3	99.9 \pm 0.4
	10	9.6 \pm 2.1	52.8 \pm 5.7
Gentiobiose	20	60.5 \pm 0.6	100.0 \pm 11.0
	10	14.3 \pm 0.6	100.0 \pm 4.5
	5	0	56.1 \pm 4.9
Isomaltose	10	17.0 \pm 8.9	39.8 \pm 3.9
Cellobiose	10	0	62.8 \pm 0.7
Maltose	10	0	57.0 \pm 0.3

^a Results of two experiments. SD, Standard deviation.

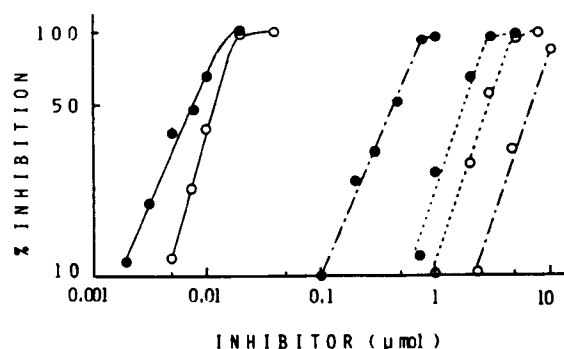


FIG. 2. Inhibition of precipitin reaction between two MABs (○; MAB a-4, ●; MAB a-84) and HSI antigen. MABs a-4 (40 μ l) and a-84 (50 μ l) were incubated with various amounts of inhibitors for 1 h at 37°C and then the final volume was adjusted to 120 μ l. After incubation at 37°C for 1 h, the mixture was kept at 4°C for 1 day. The amount of protein precipitated was determined. Symbols: —, melibiose; ---, stachyose; ···, galactose.

most strongly. Raffinose and stachyose, which contain melibiose as part of their molecules, showed less inhibition. It was surprising that inhibition by stachyose was different between the two MABs. This may mean that the predominant immunodeterminants recognized by the two MABs are the same, but a minor difference exists between the two MABs. Although melibiose, galactose- α 1,6-glucose, showed the strongest inhibition, glucose didn't inhibit the reaction at the indicated doses. It was studied carefully at higher doses whether glucose is involved in the reaction (Table 3). Gentiobiose, glucose- β 1,6-glucose, inhibited the reactions of the two MABs markedly. Isomaltose, glucose- α 1,6-glucose, inhibited the reaction of MAB a-4, even at 10 μ mol, strongly. Consequently, a specific form of glucose, possibly linked at position 6, may be involved in the precipitin reaction.

Further hapten inhibition tests were performed (Fig. 2). When 50% inhibition values were compared, it was found that inhibition by melibiose was over 200-fold stronger than that by galactose. Considering the strong inhibition by melibiose and the involvement of glucose, galactose- α 1,6-glucose seems to be the predominant immunodeterminant of the a-d-h site. When inhibition by stachyose was compared between two MABs, the difference stated above in the affinities of the two MABs for stachyose was confirmed again. This suggests that the galactose and fructose moieties of stachyose inhibit the binding of MAB a-4 to stachyose.

In this study, it was demonstrated that MABs are very useful for analysis of polysaccharide antigens (1, 9).

We thank S. Hamada and R. R. B. Russell for supplying bacterial strains.

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

LITERATURE CITED

- Ackermans, F., J.-P. Klein, F. Cormont, H. Bazin, J. A. Ogier, R. M. Frank, and J. Vreven. 1985. Antibody specificity and antigen characterization of rat monoclonal antibodies against *Streptococcus mutans* cell wall-associated protein antigens. *Infect. Immun.* 49:344-350.
- Beighton, D., R. R. B. Russell, and H. Hayday. 1981. The isolation and characterization of *Streptococcus mutans* serotype *h* from dental plaque of monkeys (*Macaca fascicularis*). *J. Gen. Microbiol.* 124:271-279.
- Brown, T. A., and A. S. Bleiweis. 1979. Chemical, immunochemical, and structural studies of the cross-reactive antigens of *Streptococcus mutans* AHT and B13. *Infect. Immun.* 24:326-336.
- Coykendall, A. L. 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on their molecular composition. *Int. J. Syst. Bacteriol.* 27:26-30.
- Fitzgerald, R. J., and P. H. Keyes. 1960. Demonstration of the etiological role of streptococci in experimental caries in the hamster. *J. Am. Dent. Assoc.* 61:9-19.
- Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard. 1977. Antibodies to major histocompatibility antigen produced by hybrid cell lines. *Nature (London)* 266:550-552.
- Gibbons, R. J., and J. van Houte. 1975. Bacterial adherence in oral microbial ecology. *Annu. Rev. Microbiol.* 29:19-44.
- Hamada, S., and H. D. Slade. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* 44:331-384.
- Kato, H., F. Ota, K. Fukui, and K. Yagawa. 1986. Monoclonal antibody to *Streptococcus mutans* type e cell wall polysaccharide antigen. *Infect. Immun.* 52:628-630.
- Linzer, R., and H. D. Slade. 1974. Purification and characterization of *Streptococcus mutans* group d cell wall polysaccharide antigen. *Infect. Immun.* 10:361-368.
- Moore, W. E. C., E. P. Cato, and L. V. H. Moore. 1985. Index of the bacterial and yeast nomenclatural changes published in the *International Journal of Systematic Bacteriology* since the 1980 approved lists of bacterial names (1 January 1980 to 1 January 1985). *Int. J. Syst. Bacteriol.* 35:382-407.
- Mukasa, H., and H. D. Slade. 1973. Extraction, purification, and chemical and immunological properties of the *Streptococcus mutans* group "a" polysaccharide cell wall antigen. *Infect. Immun.* 8:190-198.
- Okahashi, N., Y. Nishida, T. Koga, and S. Hamada. 1984. Immunochemical characteristics of *Streptococcus mutans* serotype *h* carbohydrate antigen. *Microbiol. Immunol.* 28:407-413.
- Ota, F., M. Kiso, K. Okada, H. Kato, K. Hirota, K. Fukui, M. Yasuoka, M. Ono, K. Uegaki, and Y. Morimoto. 1985. *Streptococcus mutans* serotype *b* strain (*St. rattus*, Coykendall): first isolation in Japan from human dental plaque. *Microbiol. Immunol.* 29:1005-1010.
- Prakobphol, A., R. Linzer, and R. J. Genco. 1980. Purification and characterization of a rhamnose-containing cell wall antigen of *Streptococcus mutans* B13 (serotype *d*). *Infect. Immun.* 27:150-157.
- Scherp, H. W. 1971. Dental caries; prospects for prevention. *Science* 173:1199-1205.
- Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* 30:225-420.
- Takada, K., J. Wyszomirska, and T. Shiota. 1984. Serological characterization of *Streptococcus mutans* serotype polysaccharide *g* and its different molecular weight forms. *Infect. Immun.* 45:464-469.
- van de Rijn, I., and A. S. Bleiweis. 1973. Antigens of *Streptococcus mutans*. I. Characterization of a serotype-specific determinant from *Streptococcus mutans*. *Infect. Immun.* 7:795-804.